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Ricasolia amplissima (*Lobariaceae*): one species, three genotypes and a new taxon from south-eastern Alaska

Carolina CORNEJO, Chiska DERR and Karen DILLMAN

Abstract: The genetic diversity within the foliose form of *Ricasolia amplissima* from Europe and North America was studied using molecular phylogenetic analysis of the nuclear ITS and *RPB2*, and mitochondrial SSU. Boundaries between closely related species were also examined using morphological and chemical patterns. Four species of the recently reinstated lichen genus *Ricasolia* De Not. were phylogenetically verified which necessitated a new combination, *Ricasolia japonica* (Asah.) Cornejo. Analyses suggest that the generic type taxon *R. amplissima* (Scop.) De Not. belongs to a species complex that shows two evolutionary centres, one in Europe, North Africa, Asia Minor and the Macaronesian Islands, the other from north-western North America on exposed shores of mainly forested marine islands in south-eastern Alaska, where it shows strong habitat specificity. The Alaskan lineage is very similar to the European lineage but it differs by the lack of scrobiculin and other chemical substances. It is described here as *R. amplissima* subsp. *sheiyi* Derr & Dillman.

Key words: ascomycetes, chemotypes, evolutionary significant unit, lichens, phylogenetic analysis, *Ricasolia japonica*

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Introduction

The long-established concept of the family *Lobariaceae* Chevall., comprising the genera *Sticta* (Schreb.) Ach., *Pseudocyphellaria* Vain. and *Lobaria* (Schreb.) Hoffm., has recently been revised based on a phylogenetic study which revealed 12 genera instead of the three mentioned above (Moncada *et al*. 2013). The present study follows the generic divisions proposed by Moncada and collaborators and takes a critical look at some species within the genus *Ricasolia* De Not., which was previously treated as section *Ricasolia* (De Not.) Vain. within *Lobaria* s. lat. (Yoshimura 1971). We focus particularly on the genetic diversity within foliose specimens of the generic type species *R. amplissima* (Scop.) De Not. from Europe and North America. While in the Old World *R. amplissima* is a well-known lichen of humid, nemoral habitats in Europe, North Africa, Asia Minor and the Canary Islands, three morphological variants of this species have been reported for North America: a composite specimen from northern California (Tønsberg & Goward 2001), a foliose specimen from south-eastern Alaska (Tønsberg & Goward 2001) and a dendriscocauloid specimen from Montana (McCune *et al*. 2014) (all identified as *Lobaria amplissima* (Scop.) Forssell). However, additional foliose specimens have been found in at least 23 other sites in Alaska, epiphytic on the trunks and lower branches of old-growth *Picea sitchensis* (Bong.) Carr. and *Tsuga heterophylla* (Raf.) Sarg. forests of marine beaches (Dillman 2004, 2010). In assessing the placement of the Alaskan population, however, three clear distinctions from European *R. amplissima* emerged: the lack of chemical compounds, the lack of erumpent cephalodia or the dendriscocauloid form in close proximity, and the lack of fertile apothecia.

Early attempts to identify the Alaskan material by Derr and others suggested that it could

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be closely related to one of four morphologically similar species formerly in the genus *Lobaria* s. lat.: *Lobaria japonica* (Zahlbr.) Asah. (hereafter referred to as *Ricasolia japonica*), *R. amplissima*, *R. quercizans* (Michx.) Stizenb. or *R. virens* (With.) H. H. Blom & Tønsberg. *Ricasolia amplissima* and *R. quercizans* are morphologically similar and can be indistinguishable without the analysis of secondary products. While the European *R. amplissima* produces mainly scrobiculin (Culberson 1967*a*, *b*, 1969; Huneck *et al*. 1973; Elix & Tønsberg 2006), North American *R. quercizans* contains abundant gyrophoric acid (Hale 1957; Culberson 1969; Jordan 1973). However, no lichen substances were detected by thin-layer chromatography (TLC) of *R. amplissima* samples collected in Alaska (Dillman 2010) or in the specimen from California (Tønsberg & Goward 2001). Lichen substances were not detected in European specimens of erumpent cephalodia attached to the chloromorph of *R. amplissima* (Culberson 1967*b*; James & Henssen 1976; Tønsberg & Holtan-Hartwig 1983) nor are they found in the morphologically similar East Asian *R. japonica* (Yoshimura 1971) or the European *R. virens* (Schumm 2003).

In Europe, erumpent cephalodia are known to occur as coralloid extensions on the dorsal surface of some cyanolichens (Moreau 1921; Kaule 1932; James & Henssen 1976; Tønsberg & Holtan-Hartwig 1983; Cornejo & Scheidegger 2013*a*). The dendriscocauloid forms are often called cyanomorphs when they occur sympatrically with the foliose thalli (chloromorphs) of *R. amplissima* and are believed to have become detached from the foliose thalli. Genetic tests of European material have identified the same fungal component within foliose *R. amplissima* and its dendriscocauloid form (Armaleo & Clerc 1991; Stenroos *et al*. 2003), which supports this concept. In North America, erumpent cephalodia were not detected in any of the south-east Alaskan populations, nor were dendriscocauloid forms observed in close proximity. The aforementioned tiny, composite California specimen from a dry oak savannah is the only known exception in North America where the *R. amplissima* chloromorph and dendriscocauloid form are attached to one another (Tønsberg & Goward 2001).

Tønsberg & Goward (2001) proposed treating the North American specimens as *Lobaria* (*Ricasolia*) *amplissima* but, from a biogeographical point of view, *R. japonica* or *R. quercizans* could be valid alternative species as well. An Asian common ancestor of *R. japonica* and North American material could have migrated across the Bering Land Bridge which has been frequently demonstrated to be an active pathway for the migration of seed plants, ferns, lycophytes and bryophytes between Asia and North America (Schofield 1988; Xiang & Soltis 2001; Manos & Meireles 2015; Xiang *et al*. 2015). Alternatively, North American material could share a common ancestor with *R. quercizans*. Biogeographical analyses of bryophytes and seed plants provide evidence that floristic disjunction between eastern and western North America represents the fragmentation of a once continuous mixed forest community (Schofield & Crum 1972; Xiang *et al*. 1998; Shaw 2001; Xiang & Soltis 2001; Shaw *et al*. 2003; Manos & Meireles 2015).

In the present study, we investigate the taxonomic position of the North American foliose material from Alaska under the hypothesis that it belongs to one of four closely related *Ricasolia* species from: Europe, 1) *R. amplissima* and 2) *R. virens*; North America, 3) *R. quercizans*; or Eastern Asia, 4) *R. japonica*. For this purpose, morphological and chemical patterns were investigated and a molecular phylogeny was generated based on three genetic markers: nuclear ITS and *RPB2*, and mitochondrial SSU. The taxonomy of, and phylogenetic relationships between, erumpent cephalodia and the dendriscocauloid form of this (and other) species complexes are not addressed in this analysis.

Material and Methods

The present work studies species within the reinstated genus *Ricasolia* with emphasis on the type species *R. amplissima* from Europe and North America (specifically Alaska). For this purpose, fresh material was collected during different field excursions in Alaska and the Sakhalin and Macaronesian Islands, and stored air-dried

and frozen (−20 °C). Honegger (2003) demonstrated that soft cell structures within highly hygroscopic lichens disintegrate within a few years if specimens are kept at room temperature, challenging the usual conditions applied in herbaria for the preservation of fungi (temperature 20–23 °C, humidity 40–60% RH; Ariyawansa *et al.* 2014). Thus, to slow down DNA degradation, we kept the lichen material frozen in our lichen collection until examination (hb. Scheidegger; WSL). Table 1 lists voucher information for the specimens sampled, and Table S1 (see Supplementary Material, available online) lists all sequences obtained from GenBank (NCBI suite: http://www.ncbi.nlm.nih.gov/). Figure 1 shows the known range of *R. amplissima* and allied species.

Chemical analyses were performed using standard TLC techniques (Culberson & Ammann 1979; White & James 1985). Secondary substances were extracted in acetone for *c.* 10 min at 40 °C. The extracts were spotted on pre-coated Merck silica gel F254 plates and eluted in three solvent systems: A) toluene: dioxan: acetic acid (180:45:5 ml); B) hexane: diethylether: formic acid (130:100:20 ml); and C) toluene: acetic acid (200:30 ml). For each solvent system, we used a separate plate spotted with the same extracts. The plates were examined first by UV light at 254 nm and 366 nm wavelengths. The plates were then immersed briefly in a sulphuric acid bath, air dried and heated in an oven at 100 °C for *c.* 15 min. Identification of the substances was made by comparison with known references from our laboratory.

The DNA isolation of all specimens, the polymerase chain reactions of the ITS and *RPB2* loci, and the cycle sequencing were performed as described in Cornejo & Scheidegger (2010). Additionally, we used the primer set mrSSU1 and mrSSU3R for the amplification of the mitochondrial small subunit of the RNA gene according to Zoller *et al.* (1999).

Sequences were aligned in DNA Workbench 7 (CLC bio, Aarhus, Denmark) and corrected by hand if necessary. Regions with ambiguous alignments were excluded from all analyses by processing all datasets with Gblocks 0.91b (Castresana 2000; Talavera & Castresana 2007) on the Phylogeny.fr platform (Dereeper *et al.* 2008). Phylogenetic trees were reconstructed by maximum likelihood (ML) and Bayesian statistics (based on a Markov chain Monte Carlo algorithm), using PhyML 3.0 (Guindon & Gascuel 2003; Guindon *et al.* 2010) on the ATGC platform (www.atgc-montpellier.fr) and BEAST 1.8.2 (Drummond & Rambaut 2007) on the CIPRES Science Gateway (Miller *et al.* 2010), respectively. For phylogenetic analyses, the interactive software jModelTest v.0.1.1 (Guindon & Gascuel 2003; Posada 2006, 2008) and the Akaike Information Criterion (AIC) or the corrected AICc for short samples (Akaike 1973; Hurvich & Tsai 1989) were used to select the model that best fitted our data. Bootstrap confidence values (B) were calculated for 1000 pseudoreplicates (Felsenstein 1985). To obtain the Bayesian posterior probabilities (PP), a maximum clade credibility tree was generated by analyzing the BEAST tree file in TreeAnnotator v.1.8.2 (available from http://beast.bio.ed.ac.uk/). Phylograms of both concatenated and single locus datasets were produced with TreeGraph 2 (Stoever & Mueller 2010). Detailed specifications on phylogenetic analyses are available in Cornejo & Scheidegger (2015).

Single locus trees were used to detect conflicting phylogenetic signals among the sampled markers. For this purpose, clades of the separate gene trees were examined for well-supported (≥700 of 1000 replicates) conflict between maximum likelihood phylogenies. A restricted dataset excluding specimens that were represented by only one sequence was used to concatenate the three genes in order to improve the detection of monophyly. The combined matrix contained 2405 nucleotide sites and included 37 terminal units, 29 of which were represented by three genes, and eight by two genes (Table 1; Table S1). Moncada *et al.* (2013) have shown that taxa of *Dendriscosticta* Moncada & Lücking, *Lobariella* Yoshim. and *Yoshimuriella* Moncada & Lücking belong to different closely related genera within *Lobaria* s. lat. Taxa of these genera were therefore chosen as outgroup to test the monophyly of the ingroup. Bifurcations of $B \leq 700$ and $PP \leq 0{\cdot}90$ support were considered statistically non-significant and collapsed using the software TreeGraph, with the exception of branches with ambiguous results shown on trees. A phylogenetic species was considered strongly supported if a particular lineage exhibited monophyletic patterns in a majority of sampled loci (genealogical concordance), which were not contradicted by phylogenetic patterns in other loci (genealogical non-discordance) (Taylor *et al.* 2000; Dettman *et al.* 2003, 2006).

Results and Discussion

Phylogenetic patterns within the genus *Ricasolia*

For this study, 26 ITS, 19 *RPB2* and 16 mrSSU sequences were newly sampled. Table 2 summarizes information on data matrices and the results of analyses with Gblocks and jModelTest. A few important herbarium specimens were collected a long time ago and their extracted DNA was of poor quality. This is the case for the Californian and Alaskan specimens of *R. amplissima* collected in 1999 (Tønsberg & Goward 2001) and received from HSU and BG, respectively, as well as the Slovenian specimens collected in 2006 and 2012, both received from GZU. PCR of these samples failed in most cases and only ITS sequences of GZU-5-2006 and GZU-04-2012 were obtained. Figure 2 shows the single-locus trees graphically edited with the software TreeGraph, which was used to collapse all branches with bootstrap values $B < 500$ (of 1000 replicates). Because all single-locus trees presented a concordant topology,

TABLE 1. *Voucher information for the sequenced* Ricasolia *specimens used in this study together with their GenBank Accession numbers.*

Taxon	Locality	*Collectors* and voucher numbers*	DNA-ID	GenBank Accession numbers		
				ITS	*RPB2*	mrSSU
Ricasolia amplissima	Turkey, Bolu Daglar	*Scheidegger & Scheidegger*; SCH–13790	TUY/15b	KR476692†	KC602528†	KC494183†
	USA, Alaska	*Dillman* 2008-602 (G) SCH–17017	X50	KX385118	KX385158	KC494188†
	USA, Alaska	*Dillman* 2007-10 (ALA) SCH–17019	X52_1	KX385119	KX385159	KC494187†
	USA, Alaska	*Dillman* 2007-10 (ALA) SCH–17019	X52_2	KX385120	KX385160	KX385144
	Spain, Sierra de Ayllón	*Aragón & Aroa, Otálora* 101008; SCH–17077	X58	KX385121	KC602529†	KC494182†
	Spain, Sierra de Ayllón	*Aragón & Aroa, Otálora* 081008; SCH–17078	X59	KX385122	KX385161	KX385150
	Spain, Parque Natural Hayedo de Tejera Negra	*Martínez & Otálora; Otálora* 031008; SCH– 17079	X60	KR476693†	KX385162	KX385151
	Spain, Parque Nacional Picos de Europa	*Aragón* 3123/03; SCH–17080	X61	KX385123	KX385163	KX385152
	Spain, Parque Nacional Picos de Europa	*Goyo* 3986/03; SCH–17083	X64	KX385124	KX385164	KX385153
	USA, Alaska	*Dillman* 2009-203; SCH–19900	X115	KX385125	KX385165	KX385142
	USA, Alaska	*Dillman* 2009-206; SCH–19901	X116	KX385126	KX385166	KX385143
	Portugal, Natural Park of Serras de Aire and Candeeiros	*Cornejo*; SCH–19902	X117	KX385127	KX385167	KX385145
	Algeria, National Parc of El Kala	*Abderachid*; SCH–14679	X153	KX385128	KX385168	KX385148
	Slovenia, Snežnik Mountains	*Mayrhofer* (GZU 5-2006)	CC04	KY783727	–	–
	Slovenia, Snežnik Mountains	*Mayrhofer & Spribille* (GZU 04-2012)	CC05	KY783728	–	–
R. japonica	Russia, Sakhalin	*Scheidegger, Chabanenko & Taran*; SCH–1548	RS/90	KX385129	KX385169	KX385156
	Russia, Sakhalin	*Scheidegger, Chabanenko & Taran*; SCH–1593	RS/111	KX385130	KX385170	KX385157
R. quercizans	Canada, Newfoundland	*Scheidegger*; SCH– 2932	CN/01	KX385131	KC602560†	KC494191†
	Canada, Nova Scotia	*Conway*; SCH–7345	SC/34	KX385132	KX385171	KX385154
	USA, Tennessee	*Scheidegger*; SCH–6042	USSM3/38a	KX385133	KC602561†	KC494189†
	USA, Tennessee	*Scheidegger*; SCH–6043	USSM3/39a	KX385134	KX385172	KX385155
R. virens	UK, Scotland	*Scheidegger & Scheidegger*; SCH–18427	GBX/01	KX385135	KC602584†	KC494186†
	UK, Scotland	*Scheidegger & Scheidegger*; SCH–18432	GBX/06	KX385136	KX385173	KX385147
	UK, Scotland	*Scheidegger & Scheidegger*; SCH–18435	GBX/09	KX385137	KC602585†	KC494185†
	Portugal, Azores	*Duelli*; SCH–1074	PA/b	KX385138	KC602583†	KC494184†
	Portugal, Azores	*Werth & Cornejo*; SCH–13962	TE1/05d	KX385139	KX385174	KX385149
	Portugal, Natural Park of Serras de Aire and Candeeiros	*Cornejo*; SCH–19905	X119	KX385140	KX385175	–
	Portugal, Serra de São Mamede	*Cornejo*; SCH–19906	X120	KX385141	KX385176	KX385146

* all SCH–specimens are in the frozen collection of C. Scheidegger at the Swiss Federal Research Institute WSL.
† Sequences published in Cornejo & Scheidegger (2015).

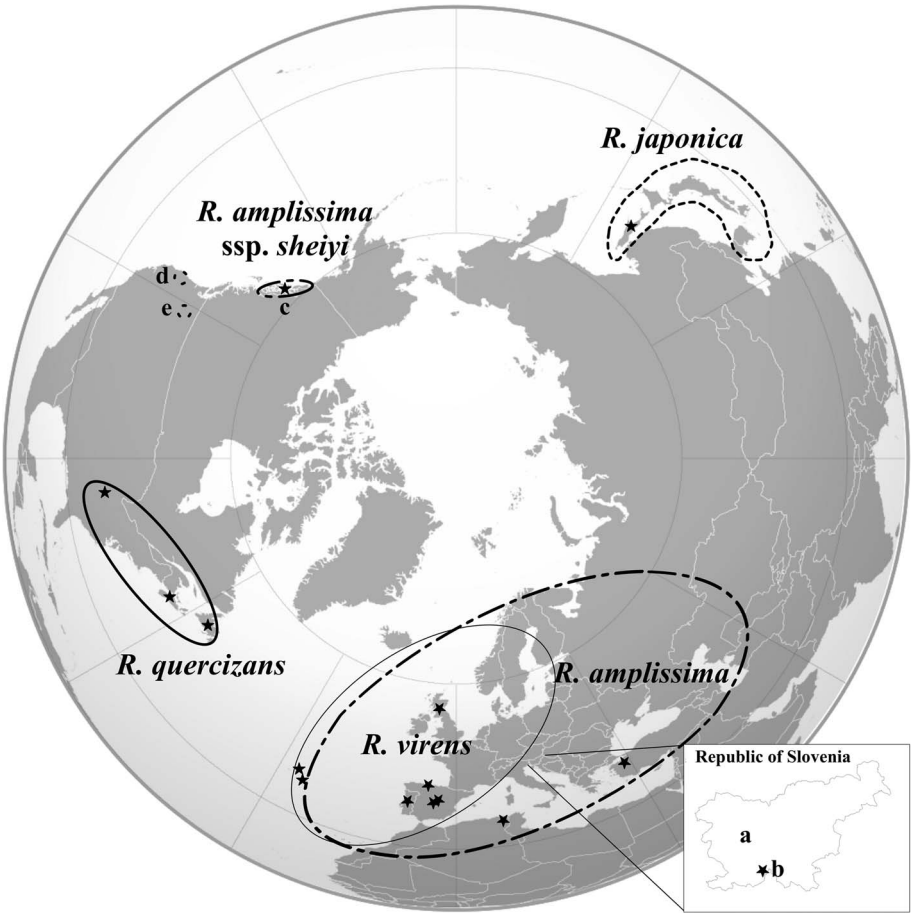


FIG. 1. Circumpolar map showing the distribution (delimited by solid and dashed lines) and collection sites (★) of *Ricasolia* species used in this study. Sites of note for *R. amplissima* include **a**, holotype, Idrija, Republic of Slovenia; **b**, Snežnik Mountains, *c.* 65 km from Idrija; **c**, foliose specimens from south-eastern Alaska; **d**, small, composite specimen, northern California, USA (Tønsberg & Goward 2001), not included in this analysis due to degraded DNA; **e**, dendriscocauloid form, Montana, USA, (McCune *et al.* 2014). (Circumpolar map: Wikimedia Commons (Rob984); map of Slovenia: d-maps.com).

multilocus analyses were performed based on a concatenated dataset.

Both the ML and Bayesian analyses of the concatenated dataset resulted in nearly identical topologies. Therefore, the ML tree was selected for the representation of data in Fig. 3 and the Bayesian tree can be found as supplementary information (see Fig. S1, available online). The phylogeny in Fig. 3 confirms the monophyly of the genus *Ricasolia*, clearly separating it from the genera *Dendriscosticta*, *Lobariella* and *Yoshimuriella* (B = 1000; PP = 0·97). This result is consistent with the three-loci phylogeny of Moncada *et al.* (2013), as well as with the ITS-phylogeny of Stenroos *et al.* (2003). Our study also confirms the systematic position of *Yoshimuriella subdissecta* (Nyl.) B. Moncada & Lücking identified by Moncada *et al.* (2013) and contradicts the naming of this species as *Ricasolia subdissecta* Nyl. based on ITS analysis (Tønsberg *et al.* 2016). Within *Ricasolia*, specimens of *R. amplissima* (B = 963; PP = 1), *R. japonica* (B = 1000; PP = 1),

TABLE 2. *Summary of molecular data matrices used in the phylogenetic analyses including the substitution model selected by jModelTest and the model effectively implemented in PhyML*.*

Locus	Number of sequences	Number of sites before / after Gblocks	Number of excluded ambiguous sites, % of total in parentheses	Number of polymorphic sites, % of total in parentheses	Substitution model jModelTest / PhyML
ITS	69	584 / 438	146 (25)	184 (42)**	TIM1+Γ / GTR+Γ
	40	–	–	72 (13)***	
RPB2	29	1126 / 1124	2 (0·2)	139 (13)**	SYM+Γ / GTR+Γ
	27	–	–	90 (6·1)***	
mrSSU	41	839 / 819	20 (3)	115 (9)**	TPM3uf+I+Γ / HKY+I+Γ
	33	–	–	39 (3·3)***	
Three-loci	37	2405	–	370 (15)**	TIM1+I+Γ / GTR+I+Γ
	31			182 (6·5)**	

* PhyML in the online ATGC-platform does not list the substitution models SYM, TIM1 or TPM3uf; therefore the next model on the AIC selection list was used for maximum likelihood analyses.
** alignment including the outgroup.
*** alignment excluding the outgroup.
Γ = Gamma distribution.

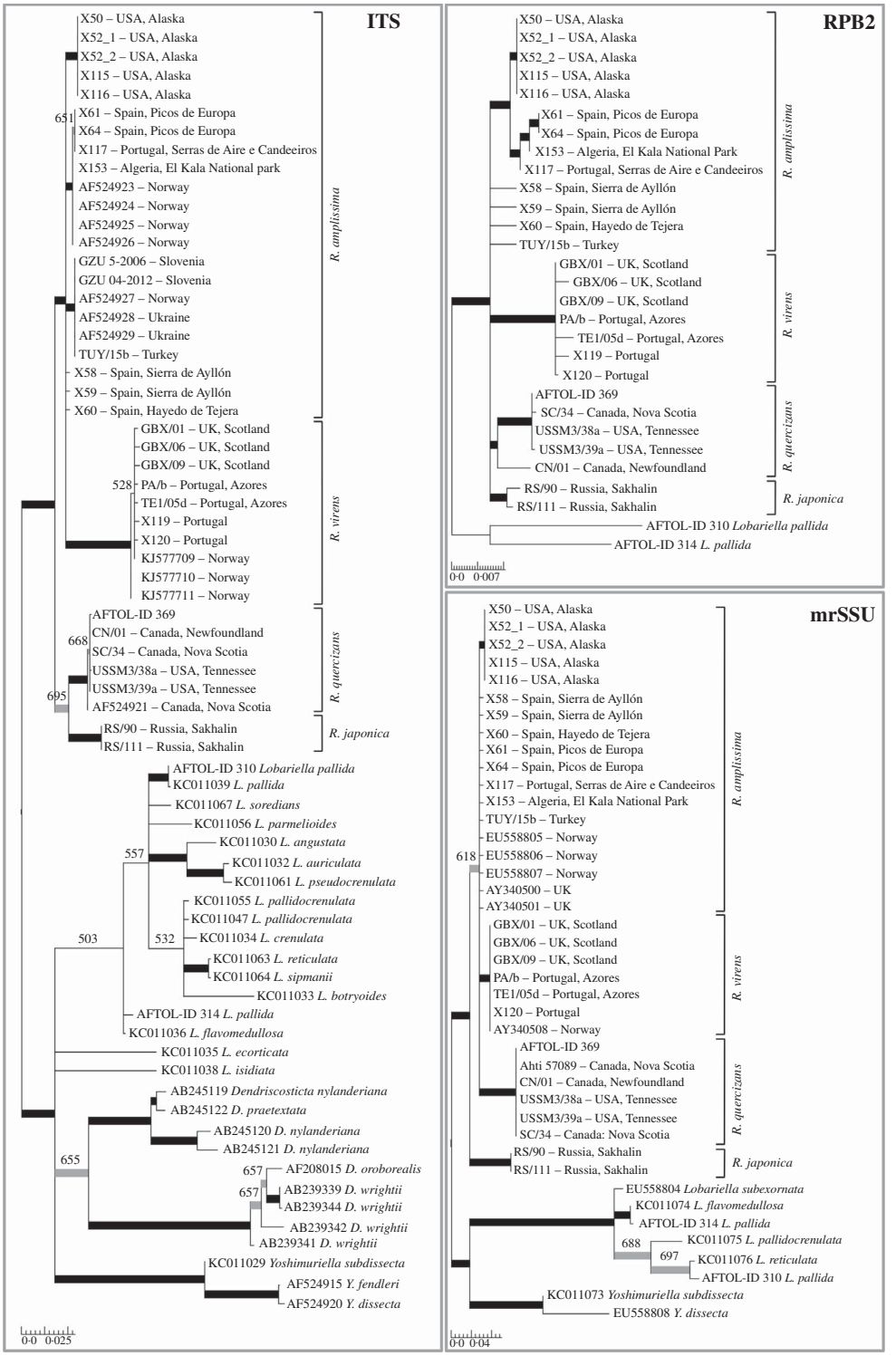
R. quercizans (B = 1000; PP = 1), and *R. virens* (B = 1000; PP = 1) compose distinct clades. However, the bifurcation of *R. amplissima* and *R. virens* had low ML bootstrap support but a high result under the Bayesian analysis. In the *R. amplissima* clade, Alaskan specimens are separated from specimens from Europe, Algeria and Turkey in different strongly supported subclades. All Alaskan specimens form a maximal corroborated subclade (B = 1000; PP = 1).

The phylogeny presented here highly corroborates a relationship at species level for specimens of *R. amplissima* from Europe and North America. However, these taxa were arranged in three statistically highly supported subclades. Surprisingly, samples from Central Spain and Turkey appear closely related to specimens from Norway (Fig. 3, subclade *a*), while other specimens from Norway are more closely related to specimens from Algeria, Portugal and northern Spain (Fig. 3, subclade *b*). The Alaskan subclade (Fig. 3, *c*) is noteworthy because, unlike the other two subclades, it has distinctive chemical, morphological and geographical patterns. These are discussed below.

Chemical characteristics

Thin-layer chromatography was used to analyze metabolites of 26 lichen samples and the resulting chemotypes are listed in Table 3, while Fig. 3 provides a short overview of the main secondary products in the specimens studied. No lichen substances were detected in *R. virens* (medulla and cortex: K−, C−, KC−, P−, UV−), confirming the results of Tønsberg *et al.* (2016). Jordan (1973) reported two chemotypes for herbarium specimens of *R. quercizans*: one containing gyrophoric acid, atranorin and two unknown substances, and the other containing gyrophoric acid, 4-0-methylgyrophoric acid, atranorin and an unknown substance. The TLC findings from our study detected only gyrophoric acid and are consistent with Culberson's chemical analyses (1969) which consistently detected gyrophoric acid in *R. quercizans* and 4-0-methylgyrophoric acid in only some specimens.

FIG. 2. Maximum likelihood phylograms of *Ricasolia* species, resulting from single locus analyses of ITS, *RPB2* and mrSSU sequences. Specimens of the genera *Dendriscosticta*, *Lobariella* and *Yoshimuriella* were used to root the trees. Bootstrap values are indicated as follows; thick black lines >700 (of 1000 replicates); thick grey lines = 600–700; thin black lines = 500–600.



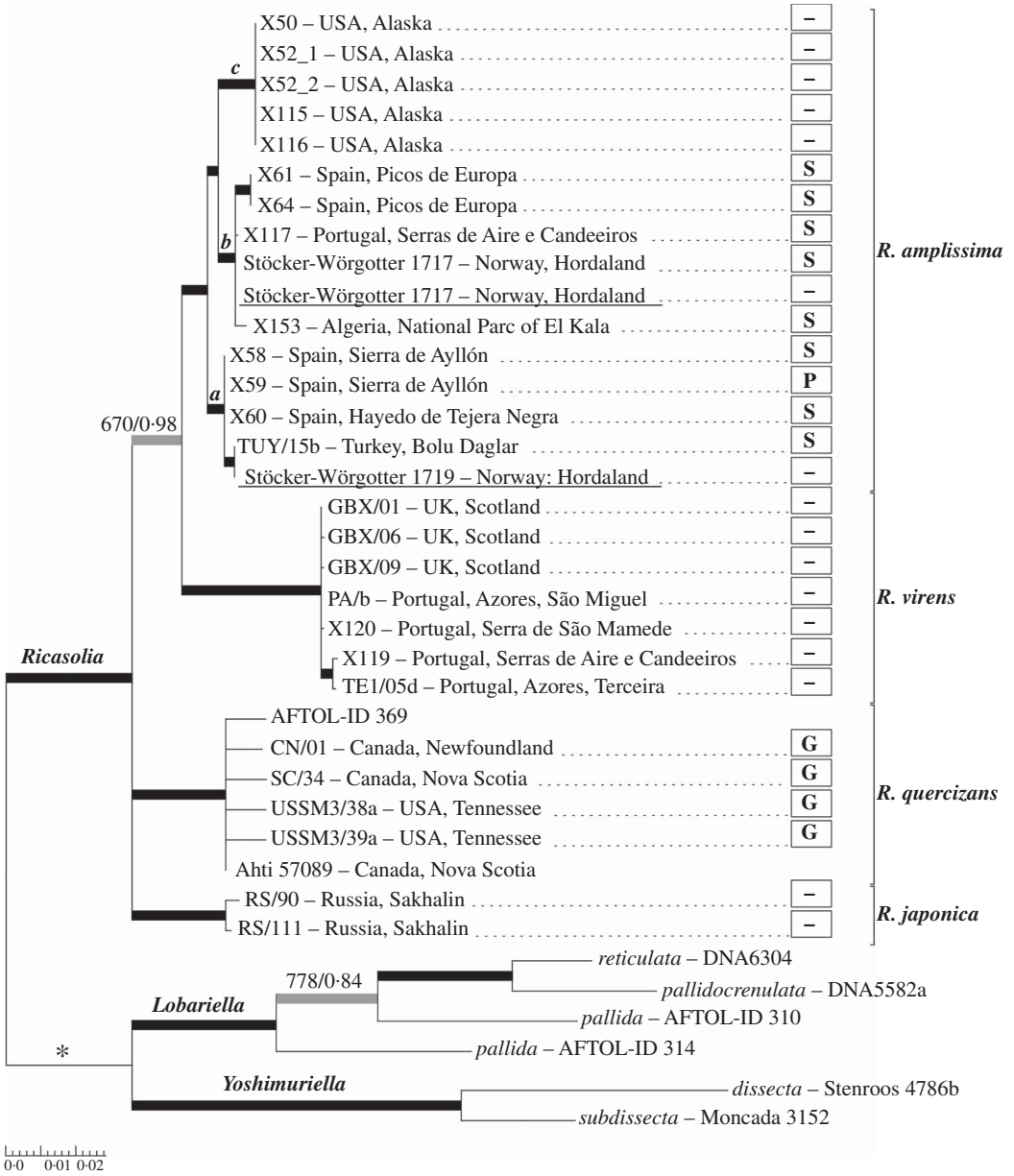


FIG. 3. Maximum likelihood phylogram of *Ricasolia amplissima* and closely related species, resulting from combined analysis of ITS, *RPB2* and mrSSU sequences. Three subclades (*a*, *b*, and *c*) were found within the monophyletic clade of *R. amplissima*. Specimens of the genera *Lobariella* and *Yoshimuriella* were used to root this tree (asterisk). Bifurcations with bootstrap values <50 were collapsed, except for two branches with ambiguous ML and Bayesian results (thick grey bifurcations). All thick black branches represent bootstrap and posterior probabilities of at least 700 (of 1000 replicates) or 0·9, respectively. Individuals known to produce a dendriscocauloid form are underlined. Major chemical compounds are indicated in rectangles: G, gyrophoric acid; P, pseudocyphellarin A; S, scrobiculin; –, no chemical compound detected.

TABLE 3. *Secondary products of European and North American* Ricasolia amplissima *and its allied species. Chemotypes of* R. amplissima *are listed as I, II, and III.*

Ricasolia	Number of specimens (*n*)	Gyrophoric acid	4-0-methylgyrophoric acid	*m*-Scrobiculin	*p*-Scrobiculin	Norstictic acid	Stictic acid	Usnic acid	Pseudocyphellarin A	Atranorin	Sample sites
amplissima chemotype I	7	—	—	++	+	—	—	—	—	(+)	Algeria; Portugal; Spain; Turkey; Slovenia
amplissima chemotype II	1	—	—	—	—	—	—	—	++	+	Spain, Sierra de Ayllón
amplissima chemotype III	5	—	—	—	—	—	(+)†	—	—	—	USA, Alaska
japonica	2	—	—	—	—	—	—	—	—	—	Russia, Sakhalin
quercizans	4	++	—	—	—	—	—	—	—	—	Canada, Newfoundland, Nova Scotia; USA, Tennessee
virens	7	—	—	—	—	—	—	—	—	—	UK, Scotland; Portugal, Azores and mainland

Key: ++ major; + minor; (+) not detected in all specimens; – absent; † detected in traces in one of five specimens.

Specimens of *R. amplissima* from Algeria, the Iberian Peninsula and Turkey contained primarily *m*-scrobiculin, *p*-scrobiculin and occasionally atranorin (chemotype I). Elix & Tønsberg (2006) reported, in addition to the substances mentioned above, pseudocyphellarin A and traces of an unknown scrobiculin derivative for specimens from Norway. On the other hand, TLC results from the northern Spain specimen X59 produced mainly pseudocyphellarin A and traces of atranorin but was negative for other chemical compounds (chemotype II). As in previous studies (Tønsberg & Holtan-Hartwig 1983; Schumm 2003; Zalewska & Bohdan 2012), atranorin was found to be an accessory substance of *R. amplissima*. However, to our knowledge, this is the first report of pseudocyphellarin A as the major chemical compound from *R. amplissima*. Culberson (1967*b*) mentioned one specimen of *R. amplissima* from Norway that showed only traces of scrobiculin with spots indicating unknown compounds; however, the author did not specify their Rf values.

In contrast with the TLC results from European material, Alaskan specimens were chemically negative (chemotype III). These findings are not surprising and were used to characterize the first species report of *Lobaria* (*Ricasolia*) *amplissima* for North America (Tønsberg & Goward 2001). It is interesting to note that TLC of the European erumpent coralloid cephalodia attached to the chloromorph and of the dendriscocauloid form did not detect chemical substances, although the corresponding chloromorph produced abundant scrobiculin (Culberson 1967*a*; James & Henssen 1976; Tønsberg & Holtan-Hartwig 1983; Stenroos *et al.* 2003: E. Stocker-Wörgötter, pers. comm.). In different parts of its range, *R. amplissima* appears to be able to produce several chemical compounds, in varying concentrations and combinations. The lichen photobiont (*Dictyochloropsis* s.l. or *Nostoc*) has been hypothesized to be one fundamental element influencing the biosynthesis of these compounds and this question has been studied in culture experiments with *R. amplissima* and *Yoshimuriella fendleri* (Tuck. & Mont.) Moncada & Lücking (Stenroos *et al.* 2003),

for example, but it has not been answered yet. The TLC results of our study question a correlation between the association with *Nostoc* or *Dictyochloropsis* strains and the inhibition of lichen substances in *R. amplissima*. Other yet unknown factors might also contribute to the variations in chemical compounds which are responsible for the presence of pseudocyphellarin A as a satellite or major substance in European specimens.

Morphological characteristics of *Ricasolia amplissima* in Europe and North America

The Alaskan populations of *R. amplissima* rarely produce apothecia but a small number of vouchers with abundant sterile apothecia have been collected (Fig. 4D; Dillman 2010). These apothecia are somewhat elevated on a constricted base and the undersides of the apothecia are rough, almost warty in texture. The thalli can be quite large, sometimes covering more than decimetre-long sections of a tree bole, and are closely pressed to the substratum. The rounded, overlapping lobes have the appearance of dried puddles of wax dripped from a candle held overhead. European populations of *R. amplissima* frequently produce fertile apothecia (Purvis *et al*. 1992). The Californian sample did not bear apothecia (Tønsberg & Goward 2001).

An important morphological difference that separates the Alaskan populations of *R. amplissima* from those in Europe is that Alaskan specimens do not appear to produce erumpent coralloid cephalodia (Dillman 2010). Instead, minute warts are occasionally present above the cephalodia close to the upper cortex. These warts could be primordial erumpent cephalodia. Tønsberg & Goward (2001) stated that a sample from the Yamani Islets of south-eastern Alaska bore globose, unbranched (not coralloid) cephalodia that measure *c.* 1 mm in diameter and we confirm this observation. These structures were assumed to represent primordial erumpent cephalodia as similar globose and warty cephalodia are also found in specimens of *R. amplissima* from Europe (Fig. 5B in Tønsberg & Goward 2001).

Some of the small warty growths, as well as mature cephalodia, observed on a number of the Alaskan samples appear to be heavily browsed by herbivores, leaving the upper surface with pits where the *Nostoc* cells were apparently mined (Fig. 4B; Dillman 2010). This supports the suggestion that at least some of the warts might be primordial erumpent cephalodia. Indeed, predation by gastropods might be one reason for the apparent lack of coralloid erumpent cephalodia in samples, as the collections are all from the lower boles of trees where terrestrial gastropods easily access lichens (Fig. 4E & F). Grazing by gastropods has also been documented on *R. amplissima* in Norway and Switzerland (Asplund *et al*. 2010; Cornejo & Scheidegger 2013*b*). Additionally, extensive grazing by snails has been reported to have a detrimental impact on populations of endangered lichen species such as *Erioderma pedicellatum* (Hue) P. M. Jørg. in Nova Scotia, Canada (Cameron 2009). In Alaska, *R. amplissima* does occur higher in the canopy but these populations were not accessible for collection and observation. Thus, it remains difficult to determine if our samples contained cephalodia that would have eventually broken through to form erumpent cephalodia. Further study is required to address this question.

In addition to the apparent lack of erumpent coralloid cephalodia on *R. amplissima* from south-eastern Alaska, the dendriscocauloid form ("*Dendriscocaulon*" per McCune & Geiser 2009) is rarely detected in exposed coastal forests at the foliose *R. amplissima* sites (Dillman 2004). However, the dendriscocauloid form can be abundant at some cyanolichen-rich sites in south-eastern Alaska, especially cool, shady, wet riparian forests near glaciers or in glacially influenced sites, where they occur on conifers and hardwood twigs such as *Salix* L. and *Alnus* Mill. (Geiser *et al.* 1998; Dillman 2010). The dendriscocauloid form has been reported frequently in North America as *Dendriscocaulon intricatulum* (Nyl.) Henss. (Goward 1994; Geiser *et al*. 1998; Lesher *et al.* 2003), *D. umhausense* (Auersw.) Degel. (≡*Polychidium umhausense* (Auersw.) Henss.) (Hale & Culberson 1966, 1970; Weber & Viereck 1967;

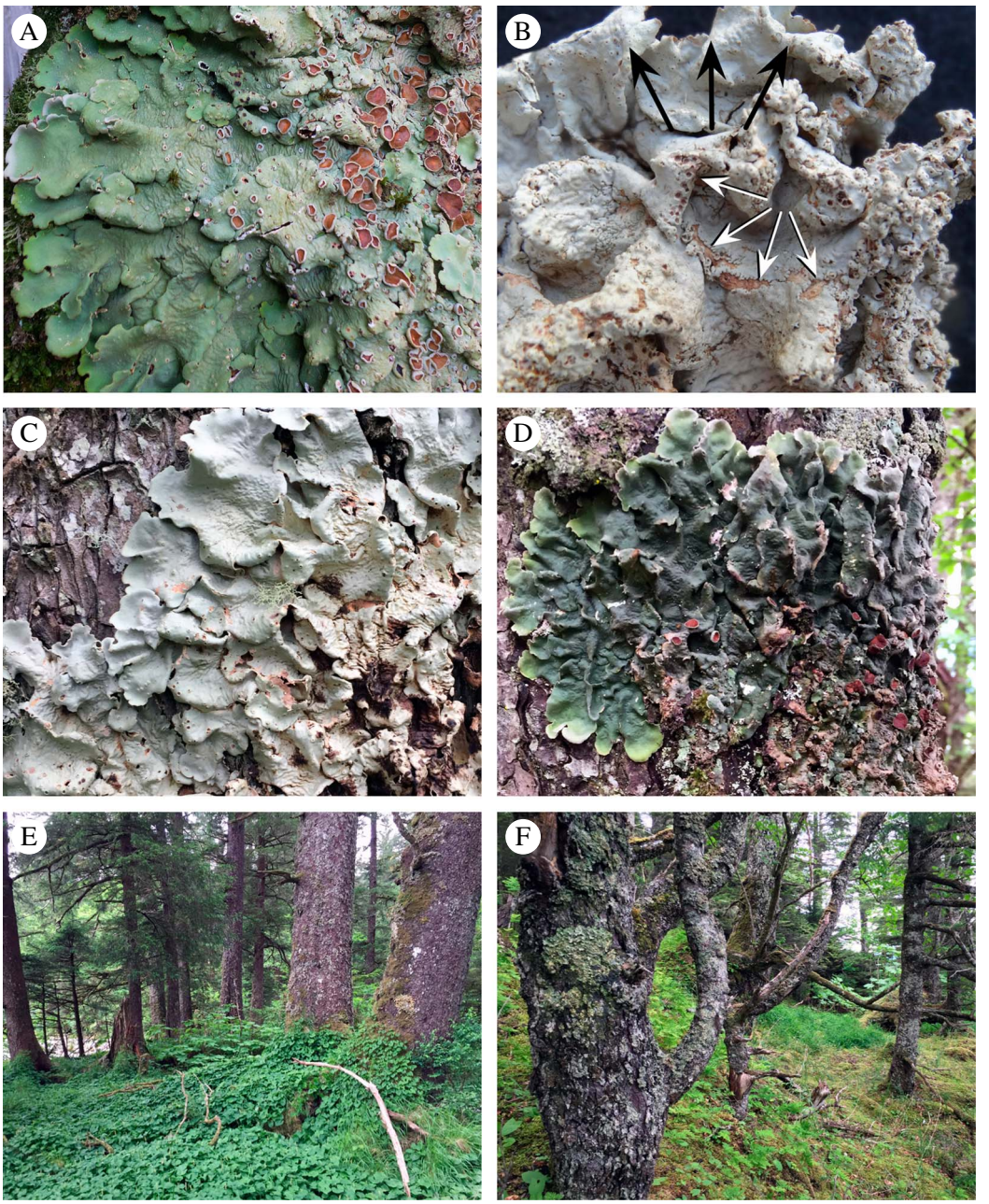


FIG. 4. *Ricasolia amplissima*. A, habit of the chloromorph from Europe (photograph: S. Stofer). B–F, *R. amplissima* ssp. *sheiyi*; B, habit of type specimen, black arrows = apical zones of the thallus, white arrows = typical wounds caused by grazing invertebrates on the upper cortex; C & D, habit, photographed in the field; E & F, field localities in Alaska: Warren Island and Sukoi Island, respectively.

Ohlsson 1973) and *Sticta oroborealis* Tønsberg & Goward, and with the chloromorph in the *Sticta wrightii* group (Tønsberg & Goward 2001). The recently reported dendriscocauloid form from Montana, verified by DNA as *Lobaria amplissima* (McCune *et al*. 2014), and other dendriscocauloid forms in Alaska and elsewhere need further study to establish the intraspecific relationship to *R. amplissima* or to other dendriscocauloid taxa of North America. Older specimens collected in North and Central America identified as *Lobaria amplissima* exist in herbaria throughout the world. These collections have not been examined in this study due to DNA degradation and will need further examination to determine if they are in the *Ricasolia* genus.

An evolutionarily significant unit of *Ricasolia amplissima* in North America

The present phylogeny suggests that Alaskan specimens are more closely related to the highly disjunct European *R. amplissima* and another European species (*R. virens*) than to eastern Asian or eastern North American taxa (*R. japonica* and *R. quercizans*, respectively) (Fig. 3). This finding reveals a geographical disjunction within *R. amplissima* which is well known in other organisms. *Parmelina quercina* (Willd.) Vain., *Letharia vulpina* (L.) Hue and *Hypogymnia hultenii* (Degel.) Krog (Culberson 1972; Printzen *et al*. 2003; Miadlikowska *et al*. 2011) are good examples of European-North American disjunct lichens, among many others (for discussion see Feuerer & Hawksworth 2007; Braidwood & Ellis 2012). In the non-vascular plants, approximately 7% of the European and 6% of the North American moss flora, and 5% of the European and 4% of the North American hepatic flora show this pattern, with a large portion of disjunction between oceanic British and Norwegian populations, and populations in western North America (Schofield 1988).

The highly supported genetic branching of the Alaskan specimens in all three loci, particularly in the slow-evolving mitochondrial DNA, suggests that Alaskan and European populations of *R. amplissima* may have little or no genetic exchange and have begun to drift independently (Figs 2 & 3). The rather long bifurcation also shows that the time of separation of the Alaskan population has been longer than that between both of the other European subclades. The combination of a distinctive chemical property, apparent reproductive isolation and subsequent genetic drift, and slow evolution of Alaskan material warrants Evolutionarily Significant Unit (ESU) status for *R. amplissima* subsp. *sheiyi* (Moritz 1995; DeWeerdt 2002). As an ESU, this geographically differentiated clade meets the criteria for biological conservation.

Taxonomic Treatment

Ricasolia De Not.

Giornale Botanico Italiano **2**(1)**:** 178 (1846); type: *Ricasolia amplissima* (Scop.) De Not., *Giornale Botanico Italiano* **2**(1)**:** 179 (1846), designated by Yoshimura, *J. Hattori Bot. Lab.* **34:** 301 (1971).

Notes. The characteristics of the genus *Ricasolia* are reported in Yoshimura (1971) under his description of the former section *Ricasolia* within the genus *Lobaria* s. lat. The present study is restricted to the nomenclatural changes for two taxa which are phylogenetically confirmed as belonging to *Ricasolia*: *R. japonica* and the new subspecies of *R. amplissima*. For the other confirmed species of *Ricasolia*, names already exist that can be used at the species level (i.e. *R. amplissima*, *R. quercizans* and *R. virens*).

Ricasolia amplissima (Scop.) De Not.

MycoBank No.: MB 404140

Giornale Botanico Italiano **2**(1): 179 (1846).—*Lichen amplissimus* Scop., *Flora carniolica* **2**: 386 (1772).—*Parmelia amplissima* (Scop.) Schaer., *Lich. Helv. Spicil.* **9**: 450 (1840).—*Sticta amplissima* (Scop.) Rabenh. *Deutschl. Krypt.-Fl.* (Leipzig) **2**: 64 (1845).—

Lobaria amplissima (Scop.) Forssell, *Bih. K. svenska Vetensk. Akad. Handl., Afd. 3* 8 (no. 3): 111 (1883); type: Italy, 1729, *Micheli, Nova Plantarum Genera* Tab. 46, top, designated as lectotype by Burgaz & Tretiach, *Taxon* **51**:765–766 (2002) (FI-M—epitype, not seen).

(Fig. 4A)

Thallus foliose, consisting of fungal, green-algal and cyanobacterial partners; composed of irregularly branched lobes with a wrinkled upper surface, especially in older parts. *Lobes* more or less imbricate, lacking soredia, isidia and true lobules but regeneration lobules can be common along predation wounds or mechanically injured areas (Cornejo & Scheidegger 2013*b*). *Photobiont Dictyochloropsis* s. lat. (Dal Grande *et al.* 2014), also contains *Nostoc* located in internal or erumpent cephalodia (Stenroos *et al.* 2003, 2006).

Chemistry. Chemotype I: *m*-scrobiculin (major), *p*-scrobiculin (sub-major), atranorin (accessory, in traces). Chemotype II: pseudocyphellarin A (major), atranorin (in traces). Chemotype II seems to be an exception and was found in only one of eight European specimens. Additionally, this specimen was genetically indistinct from other European specimens.

Range. In Europe, *R. amplissima* is a well-known lichen of humid, nemoral habitats. In Central Europe, this lichen is in decline and is cited in most Red Lists as either extinct or critically endangered (e.g. Türk & Hafellner 1999; Scheidegger & Clerc 2002; Wirth *et al.* 2011). It is also rarely found in Eastern Europe (Zalewska & Bohdan 2012). Its range extends eastward to Asia Minor and westward to the Canary Islands. In North Africa, in the Moroccan Rif and Algerian-Tunisian Khroumirie Mountains, populations occur in restricted areas with marked relief and an oceanic climate. Otte (2007) detected *R. amplissima* in the Great Caucasus in Western Asia.

Notes. Our phylogeny shows genetic variability between European and Alaskan specimens, supporting the description of a new taxon within the species *R. amplissima*. However, it also shows variability within European specimens, making the autonym *R. amplissima* ssp. *amplissima* appear paraphyletic. For that reason, it would be important to characterize the type specimen of *R. amplissima* molecularly. In 1772 Scopoli described *Lichen amplissimus* from Carniola, Slovenia: "in fago e abiete, circa Idriam in Weichenthal" (on beech and fir, in proximity to Idrija in Weichenthal; Fig. 1, site a). However, the herbarium of Scopoli was lost, and Burgaz & Tretiach (2002) designated an Italian specimen from Micheli's (1729) herbarium (FI-M) as epitype, the illustration of which is attached to *Flora Carniolica* (designated as lectotype) and described by Scopoli as "optime" in the protologue of *Lichen amplissimus*. However, DNA cannot be extracted from this epitype due to the age of the specimen, though this would be critical for the correct identification of the species type (for discussion on rules for epitypification, see Hyde & Zhang 2008; Ariyawansa *et al.* 2014). Since it is technically not feasible to characterize at a molecular level the designated Micheli epitype, we decided to include specimens from Carniola, Slovenia, collected as close to Idrija as possible (*c.* 65 km; Fig. 1, site b), to get an idea of which of the European subclades might represent the type population. Therefore, we included the specimens GZU 5-2006 and GZU 04-2012 in our dataset but the DNA was of poor quality and only the multicopy locus ITS could be amplified. The ITS tree (Fig. 2) shows the specimens GZU 5-2006 and GZU 04-2012 that group together with specimens from Norway, Turkey and Ukraine. The GZU specimens themselves are not included in Fig. 3, which was generated based on two loci at a minimum, but the most similar ITS-haplotypes form the subclade *a* in Fig. 3. Therefore, we hypothesize that subclade *a* may represent *R. amplissima* ssp. *amplissima*. It is clear that the fungal barcode is not sufficient for the characterization of *R. amplissima* ssp. *amplissima*, but this work is beyond the scope of the present study. Future research should assess the legitimacy of Micheli's epitype according to the *International Code of Nomenclature for Algae, Fungi, and Plants* (Melbourne Code; McNeill *et al.* 2012) and provide a molecular characterization of *R. amplissima* ssp. *amplissima* from fresh material, including several loci.

Early lichenologists reported *R. amplissima* from Eastern Asia; however, Yoshimura (1971) disagreed with this determination and identified Asian samples as *Lobaria japonica* (Zahlbr.) Asah.

Ricasolia amplissima subsp. sheiyi Derr & Dillman subsp. nov.

MycoBank No. MB 818300

Similar to *Ricasolia amplissima* subsp. *amplissima* but differing in the absence of scrobiculin.

Type: USA, Alaska, McDonald Island, Frederick Sound close to Petersburg, on *Picea sitchensis*, 56°50'52·25"N, 132°49'8·85"W, 5 m, 19 August 2008, *Dillman* 2008-602 (G—holotype; hb. Scheidegger SCH–17017—isotype). GenBank Accession numbers: KR476692 (ITS), KC602528 (*RPB2*) and KC494183 (mitochondrial ribosomal SSU).

(Fig. 4B–D)

Thallus foliose, forming patches or rosettes, up to 50 cm wide, often eroding and becoming blackened in the centre of the older rosettes. *Lobes* wavy-edged with rounded apices, contiguous and overlapping, up to 2·5–3·0 cm in width on the older thalli. *Upper surface* grey-white when dry, sage green when wet, young samples with a smooth surface and older specimens having an uneven wrinkly appearance. Scattered conical warts present on upper surface (some up to 1 mm in size) becoming more abundant on older thalli. *Lower surface* tomentose and light brown in older, more central portions of the thallus, light beige and less tomentose at the lobe margins, without cyphellae or pseudo cyphellae.

Apothecia rare, up to 4 mm; *disc* red-brown to dark pink; *asci* empty.

Etymology. We chose to give this new taxon a Tlingit name to honour the people who have lived in south-eastern Alaska since time immemorial. Sheiyi means Sitka spruce, which is an important tree species to the Tlingit culture and the most common substratum on which this lichen occurs. Sheiyi is pronounced ʃi:aɪ (American English: shee'-eye).

Chemistry. No lichen substances detected (chemotype III of *R. amplissima*).

Range. USA, Alaska, Alexander Archipelago, from DeLong Islands (54·9°N, 131°W) north to Canon Beach near Yakutat on the mainland (59°N, 139°W).

Habitat and ecology. In south-eastern Alaska, the exposed headlands and forested shorelines where subspecies *sheiyi* was found are almost all directly exposed to the Pacific Ocean or major bodies of sea water that are between the larger islands within the Alexander Archipelago. Many of these sites are characterized by a moderately productive forest, dominated by Sitka spruce (*Picea sitchensis*) and Pacific reed grass (*Calamagrostis nutkatensis* (J. Presl) J. Presl ex Steud.). This forest type is a narrow band (up to several hundred metres wide) parallel to the salt water edge. With increasing distance from the saltwater, the vegetation and light regimes change to a closed forest comprised predominantly of western hemlock (*Tsuga heterophylla*) and blueberry (*Vaccinium* L. ssp.) (Martin *et al*. 1995). At these sites, subspecies *sheiyi* appears to be restricted to medium and large conifer boles and branches (*P. sitchensis*, *T. heterophylla*, and rarely on *Thuja plicata* Donn ex D. Don), and the boles of older Pacific crab apple trees (*Malus fusca* (Raf.) C. K. Schneid.). The thalli examined were on the lower bole of the tree at or just above eye level, although in some locations individuals could be observed covering the bole in unreachable sections of the canopy. Only one site had thalli with abundant apothecia on more than one tree (Warren Island, Fig. 4C & E); most sites had only one or two thalli present on a single tree and usually lacked apothecia (Fig. 4D) (Dillman 2010).

In Alaska, other lichens commonly occurring with *R. amplissima* are: *Fuscopannaria laceratula* (Hue) P. M. Jørg., *Lobaria anomala* (Brodo & Ahti) T. Sprib. & McCune, *L. pulmonaria* (L.) Hoffm., *L. oregana* (Tuck.) Müll. Arg., *Parmotrema arnoldii* (Du Rietz) Hale, *Pseudocyphellaria crocata* (L.) Vainio, *P. rainierensis* Imshaug, *Ramalina farinacea* (L.) Ach., *R. menziesii* Taylor, *R. roesleri* (Hochst. ex Schaerer) Hue, *Sticta limbata* (Sm.) Ach. and *Dolichousnea longissima* (Ach.) Articus (Dillman 2004).

Notes. This description of subspecies *sheiyi* only applies to the south-eastern Alaskan foliose samples and does not include the

composite *L. amplissima* reported from California (Tønsberg & Goward 2001) or the dendriscocauloid form from Montana (McCune *et al.* 2014). We were able to examine the small Californian composite sample (*Roediger* 9/99-3), which was obtained from HSU. Because the sample was so small (chloromorph <2 cm, cyanomorph <1 cm) and old (collected in 1999), our attempts to analyze the DNA failed on both the chloromorph and the cyanomorph. At this time, the samples from California and Montana remain under *R. amplissima*.

In 1957, Hale showed morphological and chemical differences between specimens from eastern North America and those from Europe, and assigned North American material to *R. quercizans*. Although Hale (1961) also mentioned the presence of *R. amplissima* from Central America, this species has not been located again in Central America despite subsequent lichen-focused forays (Sipman & Wolf 1998; Umaña & Sipman 2002). In 1992, the second author C. Derr collected *R. amplissima* from the Sukoi Islets near Petersburg in south-eastern Alaska (*Derr* 920 ALA, OSC) (Dillman 2010). This specimen was identified by C. Derr and B. McCune as *R. quercizans* and subsequently by I.M. Brodo (Ottawa), C. Derr and B. McCune as *R. japonica*, which occurs primarily in cool-temperate deciduous forests in mountainous Japan (Yoshimura 1971). A duplicate sample was also sent to Japan for verification by I. Yoshimura, which he verified as *R. japonica*. Unfortunately, that specimen was lost in Japan when his herbarium was relocated and the record was not published. Other specimens collected since 1992 from the same location on the Sukoi Islets have been identified as *R. amplissima* by the authors, B. McCune and Dr T. Tønsberg (pers. comm.).

Additional specimens examined. **USA:** *Alaska*: Petersburg Borough, Sukoi Islets, Frederick Sound, on *Picea sitchensis*, 1992, *Derr* 920 (ALA, OSC); Kuiu Island, Explorer Basin, on *P. sitchensis*, 2003, *Dillman* 2003-639 (ALA); Coronation Island, Egg Harbor, on *P. sitchensis*, 2009, *Dillman* 2009-203 (hb. Scheidegger SCH–19900); Warren Island, Warren Cove, on *P. sitchensis*, 2009, *Dillman* 2009-206 (hb. Scheidegger SCH–19901); Windfall Islands, Tebenkof Bay, on *P. sitchensis*, 2007, *Dillman* 2007-10, (ALA; HSU; hb. Scheidegger SCH–17019); Kuiu Island, Point Ellis, on *P. sitchensis*, 2007, *Dillman* 2007-01 (ALA).

Note: lichen material used for molecular analysis is deposited in the frozen collection of C. Scheidegger at the Swiss Federal Research Institute WSL (vouchers, see Table 1).

Ricasolia japonica (Asah.) Cornejo comb. nov.

MycoBank No.: MB 817390

Lobaria laciniata subsp. *japonica* Zahlbr. *Bot. Mag. Tokyo* **41**: 324 (1927).—*Lobaria japonica* (Zahlbr.) Asah., *J. Jap. Bot.* **9**: 450 (1933).—*Lobaria amplissima var. japonica* (Zahlbr.) Zahlbr., *Cat. Lich. Univ.* **8**: 306 (1932).—*Lobaria quercizans f. japonica* (Zahlbr.) O. B. Blum, *Ukrainian Bot. J.* **32**(6): 769 (1975); type: Japan, Honshu, Daisen, *U. Faurie* 2130, designated as lectotype by Yoshimura, *J. Hattori Bot. Lab.* **34**: 306 (1971) (W—lectotype; KYO—duplicate, not seen).

Lobaria japonica f. *pallidior* Asah., *J. Jap. Bot.* **19**: 128 (1943); type: Japan, Honshu, Prov. Suruga: Subashiri, Mt. Fuji, *Y. Asahina* 64 (TNS—lectotype, designated by Yoshimura 1971).

Chemistry. No lichen substances detected.

Range. North-east Asia: Sakhalin (this study) and Japan.

Notes. This species is similar to *R. quercizans* and species of *Lobaria* s. lat. (e.g. *L. sublaevis*), which were not analyzed in this study. However, *R. quercizans* and *L. sublaevis* contain gyrophoric acid, whereas *R. japonica* lacks any secondary products.

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Supplementary Material

For supplementary material accompanying this paper visit https://doi.org/10.1017/S002428291700041X

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